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Laila Ekelund

Lung and Amniotic Fluid Phospholipids and Pulmonary Maturity in the Human Fetus

A clinical and experimental study

Malmö 1975



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LUNG AND AMNIOTIC FLUID PHOSPHOLIPIDS AND PULMONARY
MATURITY IN THE HUMAN FETUS

A clinical and experimental study

A K A D E M I S K A V H A N D L I N G

som med vederbörligt tillstånd av Medicinska fakulteten
i Lund för avläggande av medicine doktorsexamen kommer
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MATURITY IN THE HUMAN FETUS

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by

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This survey is based on the following papers:

- I. Ekelund, L., Arvidson, G. and Åstedt, B.: Amniotic fluid lecithin and its fatty acid composition in respiratory distress syndrome.
J. Obstet. Gynaec. Brit. Cwlth. 80:912, 1973.
- II. Ekelund, L., Arvidson, G. and Åstedt, B.: Lecithin-bound palmitic acid and lecithin/sphingomyelin-ratio of amniotic fluid in relation to fetal maturity. Acta Obstet. Gynec. Scand. 53:365, 1974.
- III. Ekelund, L., Arvidson, G. and Åstedt, B.: Cortisol-induced accumulation of phospholipids in organ culture of human fetal lung. Scand. J. clin. Lab. Invest. In press.
- IV. Ekelund, L., Arvidson, G., Emanuelsson, H., Myhrberg, H. and Åstedt, B.: Effect of cortisol on human fetal lung in organ culture - a biochemical electron-microscopic and autoradiographic study. Cell Tiss. Res. In press.
- V. Ekelund, L., Arvidson, G., Kullander, S. and Åstedt, B.: Effect of cortisone and ACTH on the phospholipids of human amniotic fluid and lung tissue in early gestation. Scand. J. clin. Lab. Invest. In press.
- VI. Ekelund, L., Arvidson, G., Ohrlander, S. and Åstedt, B.: Changes in amniotic fluid phospholipids at treatment with glucocorticoids to prevent respiratory distress syndrome. Submitted to Acta Obstet. Gynec. Scand.

The articles will be referred to in the text by their Roman numerals.

I N T R O D U C T I O N

In 1903, Hochheim described layers of homogeneous material adhering to the walls of some of the alveoli of the lungs in two infants who died shortly after birth. In 1925, Johnson and Meyer described the histological findings in a special type of "asphyxia neonatorum." A layer of homogeneous material - a hyaline membrane - was found in the alveoli. They considered these membranes to be aspirated amniotic fluid containing *vernix caseosa*. Thirty years elapsed before detailed clinical descriptions of respiratory distress associated with the formation of pulmonary hyaline membranes appeared (Miller and Conklin, 1955; Silverman and Anderson, 1956). Shortly after, the membrane formation was said to be a secondary, nonspecific phenomenon due to tissue damage and transudation of plasma proteins, but atelectasis was the major pathological finding of the disease (Rudolph and Smith, 1960). A specific radiographic pulmonary picture was also described (Donald and Steiner, 1953). The pathological and clinical findings constituted a special syndrome, most frequently called idiopathic respiratory distress syndrome (IRDS) or hyaline membrane disease (HMD). The criteria for the clinical diagnosis have been more or less strict. Those of Hutchison *et al.* (1964) are well defined and have been widely used. They are: expiratory grunting, tachypnea (over 60 a minute), marked and persistent sternal, subcostal, and intercostal recession during inspiration (without oxygen), and a typical radiographic picture with diffuse reticulogranu-

lar mottling in the lung fields and an "air bronchogram." A striking feature is the onset of symptoms shortly after birth. If symptoms have not appeared six to eight hours after birth, the infant is not likely to develop IRDS. Prematurely born infants are mainly at risk (Usher *et al.*, 1971). There is also evidence for an enhanced risk of IRDS after delivery by Caesarean section (Usher *et al.*, 1964; Fedrick and Butler, 1972). Intrauterine asphyxia also seems to be a predisposing factor (Bruns *et al.*, 1961). In Sweden, about 400 infants a year develop IRDS. The mortality is about 40 %, constituting one third of the total neonatal mortality.

The pathogenesis of IRDS was unknown and several aetiological factors were suggested. Thus, aspiration of amniotic fluid, asphyxia, heart failure, decreased blood volume, pulmonary hypoperfusion, disturbed autonomic regulation, deficiency of α_1 -antitrypsin and α_2 -macroglobulin, and lack of fibrinolytic enzymes were considered (for ref. see Avery and Fletcher, 1974).

In 1929, von Neergaard found that excised lungs were more difficult to inflate with air than with fluid, and postulated that the naturally high surface tension of the air/water interface was lowered by surface-active substances of unknown composition. However, many years elapsed before the presence of surface-active material in pulmonary fluid (Pattle, 1955) and lung extracts (Clements, 1957) was demonstrated. The physiological function of pulmonary surface-active material - the so called surfactant - can be defined as summarized by Farrell and Avery (1975): "an anti-atelectasis factor, located in the alveolar lining layer, which by providing

a low and variable surface tension and imparting hysteresis to the air-tissue interface, serves the 2-fold function of decreasing the pressure needed to distend the lung, and maintaining alveolar stability in a wide range of local volumes." The physiological significance of the surfactant material for lung expansion has been further shown in animal experiments by tracheal deposition of surfactant (Enhörning and Robertson, 1972; Enhörning *et al.*, 1973; Enhörning *et al.*, 1973; Enhörning *et al.*, 1975).

During recent years, the composition of pulmonary surfactant has been extensively studied (King and Clements, 1972; King and Clements, 1972; King and Clements, 1972; King *et al.*, 1973; Clements, 1973; King, 1974). According to Clements (1973), the composition of canine pulmonary surfactant is: 41 % dipalmitoyllecithin, 25 % monoenic lecithins, 5 % phosphatidylethanolamine, 4 % glycerides, 4 % phosphatidylserine + glycerol, 2 % lysolecithin, 1 % sphingomyelin, 1 % fatty acid, 9 % protein, and 8 % cholesterol. Dipalmitoyllecithin is the main surface tension lowering component, as first suggested by Klaus *et al.* (1961). The protein, according to Clements (1973), should be unique and have the function of facilitating adsorption of the surfactant. Others have questioned that the lung surfactant phospholipids exists as a part of a specific lipoprotein molecule and have suggested that this complex is an artifact caused by the methods used to isolate the surfactant (Shelley *et al.*, 1975).

The alveoli of the mature lung are lined by an epithelium consisting of macrophages and Type I and Type II cells (Bertalanffy and Le Blond, 1955). Osmiophilic

lamellar bodies are present in the Type II cells (Schulz, 1959). Macklin (1954) was the first to suggest that Type II alveolar cells produced and secreted an alveolar lining material. Buckingham *et al.* (1966) confirmed this. Although the role of the osmophilic lamellar bodies is not established, its involvement in synthesis, storage, and secretion has been claimed (Askin and Kuhn, 1971; Chevalier and Collet, 1972; Adamson and Bowden, 1973; Gil and Reiss, 1973).

In 1959, Avery and Mead demonstrated deficiency of pulmonary surfactant in lung extracts from immature infants and infants dying of hyaline membrane disease. Brumley *et al.* (1967) found reduced pulmonary lecithin content in infants who died of hyaline membrane disease. This observation was confirmed by Adams *et al.* (1970).

Pulmonary secretion contributes to the amniotic fluid (Avery and Fletcher, 1974). Helmy and Hack (1962) demonstrated the presence of phospholipids in amniotic fluid. Biezenski *et al.* (1968) reported a tendency to increase in the concentration of amniotic fluid phospholipids with advancing gestational age. These observations led to the first proposals that analysis of amniotic fluid phospholipids might be useful in predicting the risk that the newborn would develop IRDS (Graven, 1968; Scarpelli, 1968). In 1971, Gluck *et al.* reported that the lecithin/sphingomyelin ratio of amniotic fluid rose abruptly during the 35th week of gestation. They stated that IRDS could be "diagnosed" antenatally by determination of this ratio, but no cases where the infant developed IRDS were studied in that investigation.

In lambs (Brumley *et al.*, 1967) and rabbits (Kikkawa *et al.*, 1968), an increased number of Type II cells containing osmiophilic lamellar bodies, increased content of lung phospholipids, and appearance of stable alveoli were demonstrated concomitantly with advancing gestational age. The time of appearance of Type II cells and stable alveoli was also established. During studies on the initiation of parturition in lambs, Liggins (1969) noted that lambs born after cortisol infusion of the ewe were viable at an earlier age than expected. Against this background, the possibility of accelerating fetal pulmonary maturation by administration of glucocorticoids was suggested. Thus, de Lemos *et al.* (1970) found that fetal lambs treated *in utero* with glucocorticoids showed more mature lungs, demonstrated by surface tension properties, than controls. Kotas *et al.* (1971) found that rabbit fetuses treated *in utero* with glucocorticoids showed evidence of increased pulmonary maturation, as determined by pressure volume curves obtained during deflation and according to surface tension properties of lung extracts. Wang *et al.* (1971) studied the same material of lungs morphologically and demonstrated accelerated appearance of osmiophilic lamellar bodies in the glucocorticoid-treated rabbit fetuses. The same observation was made by Kikkawa *et al.* (1971). Motoyama *et al.* (1971) found an earlier appearance of pulmonary surfactant and also electron-microscopic evidence for accelerated lung maturation in rabbits treated *in utero* with cortisol. The enhanced survival of prematurely delivered rabbits after cortisol injection 48 hours before parturition was observed by Taeusch *et al.* (1972). They noted that the survival was correlated with the degree of maturity of the lung, and that it was significantly increased after cortisol treatment. In lung

slices from glucocorticoid-treated rabbit fetuses, Farrell and Zachmann (1973) demonstrated elevated lecithin content in the lung tissue and increased lecithin synthesis *de novo* via cytidine diphosphate choline.

The results of these investigations supported the suggestion that the administration of glucocorticoids accelerated the fetal pulmonary maturation in lambs and rabbits.

P U R P O S E O F T H E I N V E S T I G A T I O N

- 1 To study the composition of human amniotic fluid phospholipids and its relation to gestational age and to fetal pulmonary maturity and development of IRDS (papers I, II).
- 2 To investigate the biochemical and morphological effects of glucocorticoids on human fetal lung *in vitro* (papers III, IV) and *in vivo* (paper V).
- 3 To evaluate the clinical value of amniotic fluid phospholipid analysis in high risk pregnancies treated with glucocorticoids to prevent IRDS (paper VI).

Organ culture

Fetal lung tissue fragments were washed in Parker 199 culture medium (SBL, Stockholm) and cut into pieces about 1 mm^3 . The explants were placed on slices of gel foam (Spongostan - Ferrosan, Malmö), four explants per slice. These were then put into Carrel flasks, three slices in each. Two ml of a culture medium consisting of Parker 199/human serum, 8:2 (v/v), was added to each flask. From each fetal lung, 20 flasks were prepared. To each of ten flasks, $2 \mu\text{g}$ hydrocortisone-21-acetate (Sigma, St. Louis) in $10 \mu\text{l}$ ethanol was added. The remaining ten flasks, with $10 \mu\text{l}$ ethanol added, served as controls. The flasks were incubated for 1-6 days at 37°C . [$\text{Me}-^3\text{H}$]-Choline chloride in ethanol solution (Radiochemical Centre, Amersham; specific radioactivity 16.5 Ci/mmol) was added 12-24 hours before the termination of the experiment. To each flask, 5 or $10 \mu\text{Ci}$ in 5 and $10 \mu\text{l}$ ethanol, respectively, was added. After culture, in most of the experiments, one explant from each flask was fixed for electron microscopy and autoradiography. The remaining explants from each of five flasks belonging to the same group were pooled, yielding two samples of cortisol-treated tissue and two control samples. These explants were immediately frozen at -70°C until analysed.

Electron microscopy

The explants were divided into small fragments and immersed in the fixing fluids. Routine fixation was usually performed at $+4^\circ\text{C}$ in 2.5 % glutaraldehyde buffered in phosphate pH 8.1 according to Sørensen. It was fol-

lowed by a post-fixation in 1 % osmium tetroxide buffered in the same vehicle as for the first fixation. After dehydration, embedding followed in Vestopal W (Ryter and Kellenberger, 1958) or Araldite (Glauert *et al.*, 1965). One-micron sections were examined in phase-contrast microscope or stained for light microscopy with Richardson's azure II and methylene blue. Sections for electron microscopy were cut on a LKB Ultratome. "Staining" was performed mainly *en bloc* in 0.5 % uranyl acetate and 1 % phosphotungstic acid, or on sections with lead citrate or uranyl acetate. Philips EM 300 and Zeiss EM 10 electron microscopes were used.

Autoradiography

The lung explants were fixed in glutaraldehyde buffered with sodium cacodylate and osmified and embedded in Vestopal W, as mentioned above. Sections, 1 μ thick, for light microscopy and ultrathin, carbon-coated sections for electron microscopy, were covered with Ilford L 4 liquid nuclear emulsion; in the former case, by the dipping method, in the latter, by the loop method after previous dilution of the emulsion with distilled water (1:1). After 1-2 weeks' exposure, the light microscope autoradiographs were developed in Kodak D 19 (5 min. 18°C), briefly rinsed in distilled water, and fixed in Kodak F 24 (6 min. 18°C). They were stained through the film in Richardson's azure II and methylene blue. The electron microscope autoradiographs, after 1-2 months' exposure, were developed in Kodak D 19 (2 min. 20°C), rinsed in distilled water (30 sec.), fixed in newly made, filtered 15 % $\text{Na}_2\text{S}_2\text{O}_3$ (3 min. 20°C), and finally washed in distilled water.

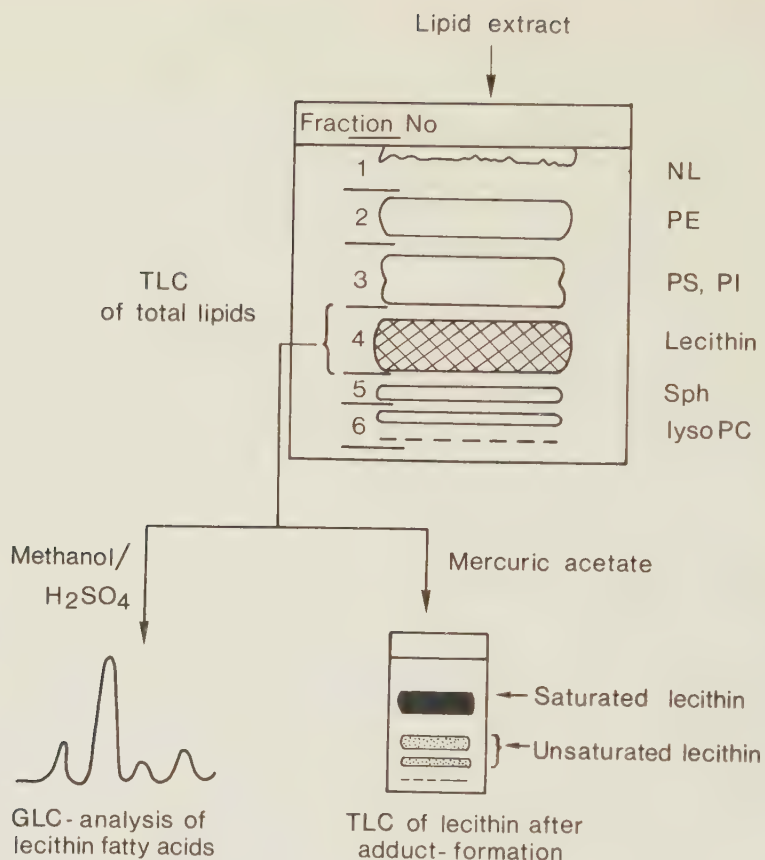


Fig. 1. General analytical procedure. The quantity of different phospholipid fractions was determined by phosphorus analysis. In the organ culture experiments the isotope content of [*Me*-³H]choline-labelled lecithin was determined by liquid-scintillation counting. Fraction No:s 1-6 refer to Tables I and III of paper I. Abbreviations: NL, neutral lipids; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; Sph, sphingomyelin; lysoPC, lysophosphatidylcholine; TLC, thin-layer chromatography; GLC, gas-liquid-chromatography.

Phospholipid analysis

Fig. 1 presents a schematic survey of the analytical procedure.

The lipids were extracted with chloroform-methanol, and the phospholipids were separated by thin-layer chromatography (Skipsky *et al.*, 1964). The phospholipid fractions were isolated quantitatively (Arvidson, 1968) and their P-contents were determined (Chen *et al.*, 1956).

The fatty acid composition and the isotope-content of the lecithin fraction were determined by gas-liquid chromatography and by liquid-scintillation counting, respectively.

The lecithin fraction was treated with mercuric acetate in methanol, as described by Mason *et al.* (1972). This converts the double bonds of lecithins containing unsaturated acyl chains into their mercuric adducts, leaving saturated lecithins intact. The reaction products were separated by thin-layer chromatography on silica gel with chloroform/methanol/ammonia, 60:30:5 (v/v), as mobile phase. Unreacted, fully saturated lecithins and the mercuric adducts of the unsaturated lecithins were isolated separately and quantitatively extracted from the silica gel according to Arvidson (1968). The isotope- and mass-distribution between these fractions was then determined. Fatty acid analysis showed that saturated fatty acids accounted for ≥ 95 % of the fatty acids in the lecithin fractions identified as saturated. The recovery of radioactivity after adduct-formation was 88.1 ± 11.9 %, and after thin-layer chromatography, 88.9 ± 14.6 % (mean value $\pm$ SD for 11 samples). The fatty acid composition of

the reconstituted lecithins recovered after mercuric adduct-formation and thin-layer chromatography agreed well with the fatty acid composition of the original unfractionated lecithins (Table I). These data indicate that saturated and unsaturated lecithins were uniformly recovered.

TABLE I Fatty acid composition of original lecithins and reconstituted lecithins recovered after mercuric adduct formation and subsequent fractionation by thin-layer chromatography. The fatty acid composition of the reconstituted lecithins was calculated from the mass-distribution between saturated and unsaturated lecithins and the fatty acid composition of these fractions.

Mean values $\pm$ SD for 13 different samples of lung lecithins are shown.

Fatty acid	Lecithin sample	
	Unfractionated	Reconstituted
	Mol %	
Myristic	1.0 $\pm$ 0.5	1.1 $\pm$ 0.5
Palmitic	73.0 $\pm$ 3.6	72.9 $\pm$ 5.5
Stearic	11.1 $\pm$ 1.1	11.2 $\pm$ 1.4
Oleic	14.7 $\pm$ 3.8	14.1 $\pm$ 5.2
Linoleic	0.4 $\pm$ 0.7	0.6 $\pm$ 0.9

Protein determinations

The lipid-free residue of the lung tissue explants was dissolved in M NaOH, and the protein was then determined according to Lowry *et al.* (1951).

M A T E R I A L A N D R E S U L T S

(Papers I-VI)

I AMNIOTIC FLUID LECITHIN AND ITS FATTY ACID COMPOSITION IN RESPIRATORY DISTRESS SYNDROME

Amniotic fluid was obtained 0.5 to 5 hours before parturition from 853 consecutive women. The infants of nine of these pregnant women developed IRDS or HMD. Amniotic fluid from these pregnancies was analysed. For comparison samples obtained from 15 women in the 35th week of gestation and from 80 full-term pregnancies were analysed. At amnioscopy, the lower pole of the amniotic sac was visualized and punctured, and the amniotic fluid was collected via the cannula directly into a centrifuge tube. From women delivered by Caesarean section, amniotic fluid was obtained by direct puncture of the uterus. The samples were immediately centrifuged and the supernatants were decanted and stored at -20°C until analysed.

The concentration of total phospholipids of amniotic fluid from those patients whose infants developed IRDS was significantly lower than in the full-term group ($p < 0.001$) and also lower than in the group with a gestational age of 35 weeks ($p < 0.01$). The molar lecithin/sphingomyelin ratio in the IRDS group was significantly lower than that in the 35-week group and in the full-term group (mean 1.60 ± 0.49 , 6.15 ± 3.36 , 12.18 ± 6.95 , resp.; $p < 0.001$).

Analysis of the fatty acid composition of the lecithin fraction showed that palmitic acid was the predomi-

nant fatty acid in all three groups, but the level was significantly lower in the IRDS group ($p < 0.001$) and the 35-week group ($p < 0.01$) than in the full-term group.

II LECITHIN-BOUND PALMITIC ACID AND LECITHIN/SPHINGOMYELIN RATIO OF AMNIOTIC FLUID IN RELATION TO FETAL MATURITY

The clinical material consisted of 92 samples of amniotic fluid obtained from 89 pregnancies throughout the third trimester terminating in the birth of healthy children. Thirty of the pregnancies were complicated. The complications were: Rh-immunization ($n=11$), toxicosis ($n=8$), suspected placental insufficiency ($n=3$), abruptio placentae ($n=5$), placenta praevia ($n=2$), and diabetes mellitus ($n=1$). The samples were collected as described in paper I, except in two cases of Rh-immunization, where repeated transabdominal amniocentesis was performed.

The proportion of palmitic acid in the lecithin and the molar lecithin/sphingomyelin ratio were determined.

The proportion of palmitic acid in the lecithin increased with advancing gestational age up to 39-40 weeks, when it flattened off and then tended to decline slightly. The values found for complicated pregnancies did not deviate from those found for normals. Also the molar lecithin/sphingomyelin ratio increased with advancing gestational age, but each week showed a marked variance. The values found for complicated pregnancies did not differ significantly from those found for the normals.

III CORTISOL-INDUCED ACCUMULATION OF PHOSPHOLIPIDS IN ORGAN CULTURE OF HUMAN FETAL LUNG

Lungs were taken from human fetuses with a crown-heel length of 17, 19, and 24 cm. The fetuses were obtained at legal abortions performed on socio-medical grounds by hysterotomy on physically healthy women. Organ cultures (ten flasks with and ten flasks without cortisol from each fetal lung) were prepared and then incubated for 6 days at 37°C. [*Me*-³H]Choline chloride was added 24 hours before termination of culture. To each flask, 5 µCi was added. After culture, the explants from five flasks belonging to the same group were pooled, yielding two samples of hormone-treated tissue and two control samples. The explants were immediately frozen and stored at -70°C until analysed 3-4 hours later.

Fig. 2 shows the obtained results. The total phospholipid content per mg tissue protein in the explants incubated with cortisol increased by 56-81 % compared with the controls. Lecithin accounted for 62-69 % of the phospholipid increment in the cortisol-treated explants, whereas only 44.2 % of the total phospholipids in the control incubation consisted of lecithin. The incorporation of [*Me*-³H]choline into the lecithin in the explants incubated with cortisol increased by 118-224 % of that of the controls. The effects of cortisol on total phospholipids, lecithin, and incorporation of choline were significant ($p < 0.001$) at each studied gestational stage and also independent of this parameter ($p > 0.05$ for interaction).

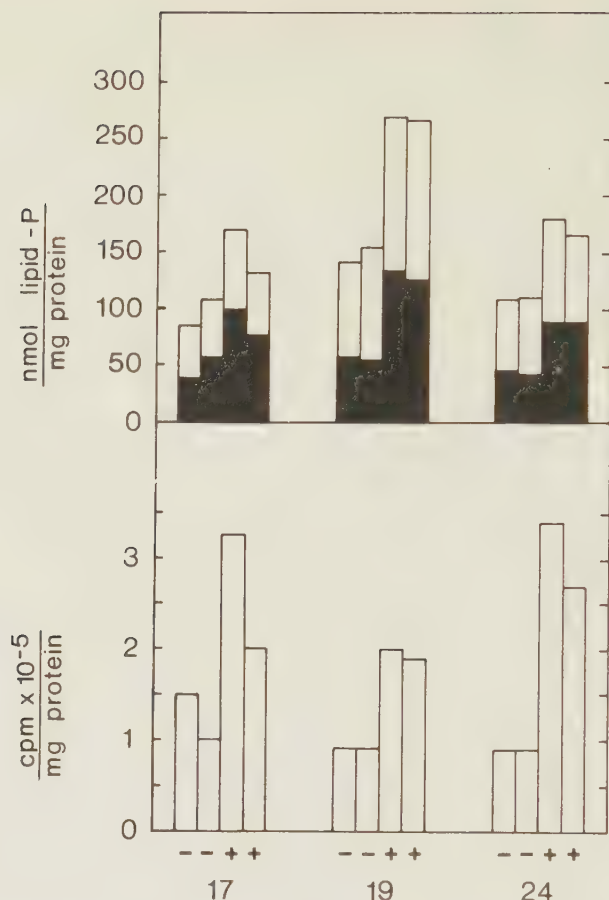


Fig. 2. The upper part shows the tissue-concentration of lipid-P. Solid bars signify lecithin; open bars, remaining phospholipids. The lower part of the figure shows the incorporation of [Me-³H]choline into the lecithin fraction. Each bar represents pooled tissue (approx. 100 mg) from 5 culture flasks. - and + refer to the addition of cortisol. The numbers below are the crown-heel length (cm) of the fetuses from which the lung explants were obtained.

IV EFFECT OF CORTISOL ON HUMAN FETAL LUNG IN ORGAN CULTURE - A BIOCHEMICAL, ELECTRON-MICROSCOPIC, AND AUTORADIOGRAPHIC STUDY

Lungs were taken from human fetuses with a crown-heel length of 11-27 cm. The fetuses were obtained at legal abortions performed on socio-medical grounds by hysterotomy on physically healthy women. The lungs were dissected and tissue fragments from the upper lobes were immediately fixed for electron microscopy. Organ cultures were prepared from the same part of the lung. From each lung, 20 flasks were prepared, ten with and ten without cortisol. The flasks were cultured for 1-6 days at 37°C. [$Me-^3H$]Choline chloride (10 μ Ci to each flask) was added 12-24 hours before termination of culture. After culture, one explant from each flask was fixed for electron microscopy and autoradiography. The remaining explants from each of five flasks belonging to the same group were pooled, yielding two samples of cortisol-treated tissue and two control samples. These explants were immediately frozen and stored at -70°C until analysed.

In noncultured and cultured control-explants from fetuses with a crown-heel length of 16-18 cm, small lamellar bodies were occasionally observed. In contrast, explants incubated with cortisol for four or six days showed numerous large osmiophilic lamellar bodies. In the explants from fetuses with a crown-heel length of 20-27 cm, well formed osmiophilic lamellar bodies were frequently found in the noncultured lung tissue and the cultured controls. In explants incubated with cortisol for two to four days, the lamellar bodies again increased in number and size.

TABLE II Concentration of phospholipids in human fetal lung tissue cultured for four days with and without cortisol

Crown-heel length of the fetus (cm)	nmol lipid-P/mg protein					
	Total phospholipids minus lecithin		Unsaturated lecithins		Saturated lecithins	
	-	+	-	+	-	+
16	52	86	35	46	21	29
	47	67	31	46	19	29
	$\Delta = 27^{**}$		$\Delta = 13^{**}$		$\Delta = 9$ NS	
20	73	101	27	48	36	88
	80	110	30	53	40	99
	$\Delta = 29^{**}$		$\Delta = 22^{***}$		$\Delta = 56^{***}$	
22	55	81	27	40	45	86
	65	77	30	43	49	92
	$\Delta = 19^*$		$\Delta = 13^{**}$		$\Delta = 42^{***}$	
23	113	111	29	37	34	54
	108	125	29	47	34	68
	$\Delta = 8$ NS		$\Delta = 13^{**}$		$\Delta = 27^{***}$	

- and + refer to the addition of cortisol. The amounts of saturated and unsaturated lecithins were calculated from the amount of total lecithins and the mass-distribution between saturated and unsaturated lecithins which was determined on the pooled lecithin from each pair of samples.

Δ is the difference between the means of the duplicate values for cortisol-treated and control explants. For each of the three parameters studied the standard deviation of Δ was estimated jointly from all the crown-heel lengths. The statistical significance of Δ is indicated as follows:

* $0.001 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$;
NS, not significant.

Table II lists the concentration of unsaturated and saturated lecithins and of the remaining phospholipids in explants incubated with or without cortisol for four days. The results show that, compared with the controls, the total concentration of phospholipids increased in the explants incubated with cortisol. In the explants from the fetuses with a crown-heel length of 20-23 cm,

the largest relative increase occurred in the saturated lecithins, whereas in the explants from the fetus with a crown-heel length of 16 cm, the relative increment was approximately the same for unsaturated and saturated lecithins and the remaining phospholipids (Fig. 3).

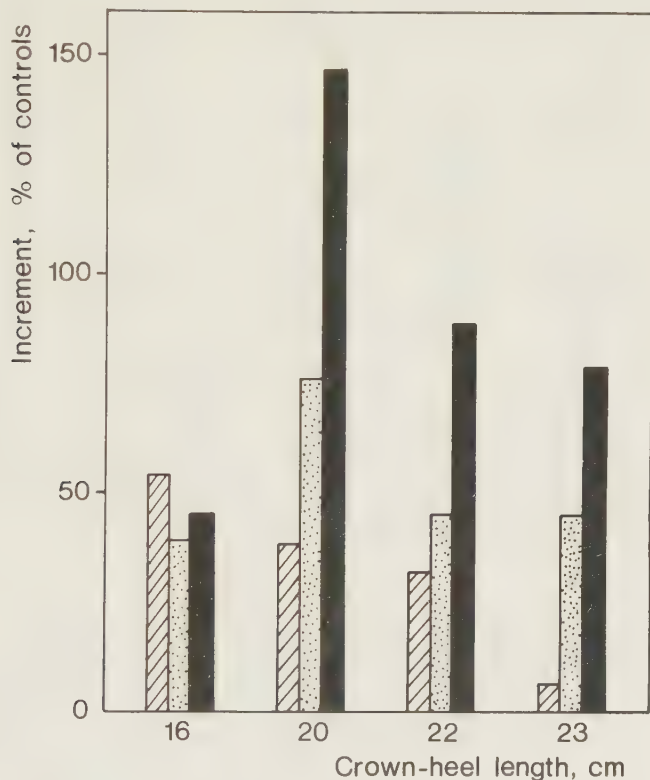


Fig. 3. Relative increment in the cortisol-treated explants of total phospholipids minus lecithin $\square$, unsaturated lecithins $\square$, and saturated lecithins $\blacksquare$. The relative increment was calculated from the Table I data according to the expression:

$$\frac{M_T - M_C}{M_C} \times 100,$$

where M_T and M_C are the means of the duplicate values of cortisol-treated and control explants, respectively.

TABLE III Incorporation of [$Me-^3H$]choline into the lecithins of human fetal lung tissue cultured for four days with and without cortisol

Crown-heel length of the fetus (cm)	cpm x 10^{-3} /mg protein			
	Unsaturated lecithins		Saturated lecithins	
	-	+	-	+
16	263	326	162	289
	262	433	224	289
	$\Delta = 117^*$		$\Delta = 96^*$	
20	122	297	143	446
	121	332	141	459
	$\Delta = 193^{**}$		$\Delta = 310^{***}$	
22	130	153	135	375
	94	257	153	386
	$\Delta = 93$ NS		$\Delta = 236^{***}$	
23	92	222	99	332
	96	295	113	461
	$\Delta = 165^{**}$		$\Delta = 290^{***}$	

- and + refer to the addition of cortisol. The definition of Δ and its statistical evaluation is given in Table I.

Table III gives the incorporation of [$Me-^3H$]choline into the lecithins of the explants. The incorporation increased in all experiments in which the explants were incubated with cortisol but was more pronounced in the explants from the fetuses with a crown-heel length of 20-23 cm. The incorporation of choline into saturated lecithins increased relatively more than the incorporation into unsaturated lecithins (Fig. 4).

In explants examined from a fetus with a crown-heel length of 26 cm, light microscope autoradiographs showed accumulation of silver grains in alveolar epithelial

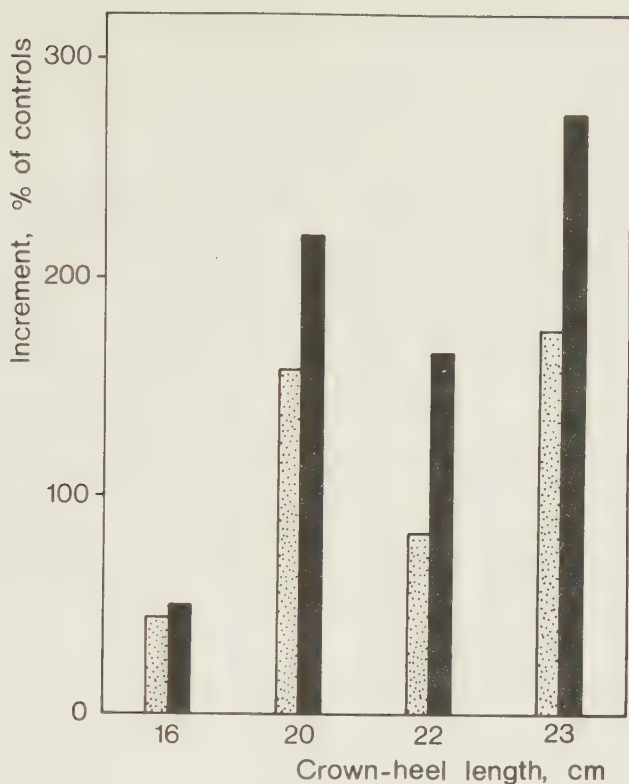


Fig. 4. Relative increment in the cortisol-treated explants of choline incorporation into unsaturated lecithins [▨] and saturated lecithins [■]. The relative increment was calculated from the Table II data according to the expression:

$$\frac{M_T - M_C}{M_C} \times 100$$

(for explanation, see Fig. 3).

cells in the cortisol-treated explants, whereas in the control explants, the grains were diffusely scattered. Electron-microscopic autoradiographs gave the same result and, moreover, revealed that in the cortisol-treated explants the grains were predominantly associated with the osmophilic lamellar bodies.

V EFFECT OF CORTISONE AND ACTH ON THE PHOSPHOLIPIDS OF HUMAN AMNIOTIC FLUID AND LUNG TISSUE IN EARLY GESTATION

Fifteen physically healthy women who had received permission for legal abortion on socio-medical grounds in the 12th-24th week of pregnancy were given either cortisone acetate or ACTH. They were informed about the purpose of the investigation and had given their full consent. Previous to abortion, performed by abdominal hysterotomy, ten patients (15th-24th week of pregnancy) received 50 mg Cortone^R (MSD) per os four times daily and five patients (17th-24th week of pregnancy) 40 IU Reacthin^R (Leo) i.m. twice daily for three days. On the fourth day, they received 200 mg Cortone^R or 40 IU Reacthin^R i.m., respectively. The hysterotomy was performed about four hours later. Eight additional patients (12th-24th week of pregnancy) served as controls.

Amniotic fluid was obtained by direct puncture of the exposed amniotic sac, and the fetus was then removed. Tracheotomy was performed on the fetus, a plastic catheter being introduced. The airways were rinsed with 3 ml of physiological saline by filling and aspirating ten times. The lungs were then immediately dissected out. The amniotic fluid samples were centrifuged, and the supernatants and also the alveolar washings and the lungs were frozen and stored at -20°C until analysed.

The lung washings contained no measurable amounts of phospholipids. The molar lecithin/sphingomyelin ratio of the amniotic fluid showed no significant differences in the three groups. In the lung tissue, the proportion of

palmitic acid in the lecithins was significantly higher ($p < 0.001$) in the cortisone-group than in the controls and tended to be correlated to gestational age ($r = 0.75$; $p < 0.05$). Fig. 5 shows the individual values for the percentage of palmitic acid. The amount of oleic acid was significantly lower ($p < 0.001$) than in the controls. In the ACTH-group, the proportion of these fatty acids was found between those of the cortisone-group and the controls, but the deviation was not significant.

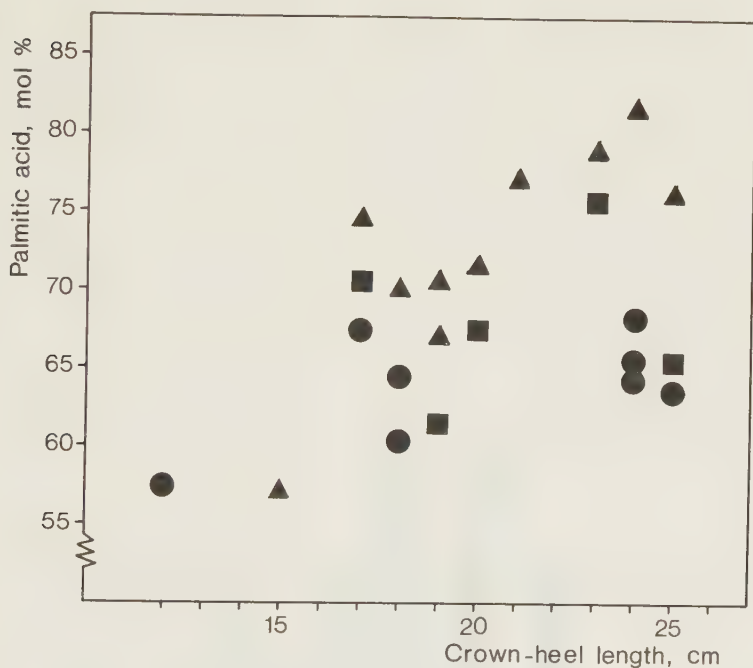


Fig. 5. Individual values of the percentage of palmitic acid in the lung lecithin.

Cortisone ▲ ACTH ■ Controls ●

VI CHANGES IN AMNIOTIC FLUID PHOSPHOLIPIDS AT TREATMENT WITH GLUCOCORTICOIDS TO PREVENT RESPIRATORY DISTRESS SYNDROME

Amniotic fluid was obtained by transabdominal amniocentesis in 51 women in the 29th-36th week of pregnancy. All were hospitalized because of pregnancy complications. The amniotic fluid lecithin/sphingomyelin ratio was determined on the day of amniocentesis. When this ratio was < 2.2 , every second patient was given 6 mg fast-acting betamethasone disodium phosphate and 6 mg slow-acting betamethasone acetate (Celestona Bifas^R, Schering corp.) i.m. daily for three days. On the fifth day, all patients underwent a second amniocentesis with subsequent analysis of the phospholipids.

The lecithin/sphingomyelin ratios of the amniotic fluid samples obtained at the first amniocentesis were < 2.2 in 28 of the patients (Fig. 6), 14 of whom were

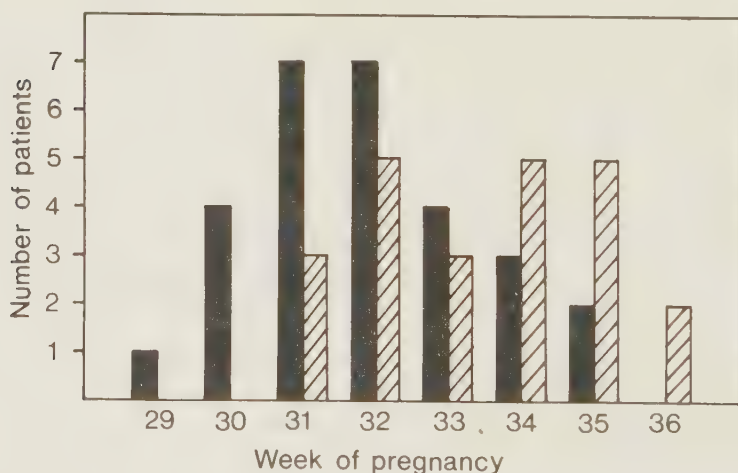


Fig. 6. Number of patients in each week of gestation with a lecithin/sphingomyelin ratio < 2.2 (■) and > 2.2 (▨), in amniotic fluid obtained at the first amniocentesis.

given Celestona Bifas^R. The remaining 14 patients served as controls. In amniotic fluid obtained at the second amniocentesis, the lecithin/sphingomyelin ratio was > 2.2 in 29 % of the patients in the betamethasone-treated group, and in 7 % of the controls (Fig. 7). The percentage of palmitic acid in the lecithin increased concomitantly with increasing lecithin/sphingomyelin ratio.

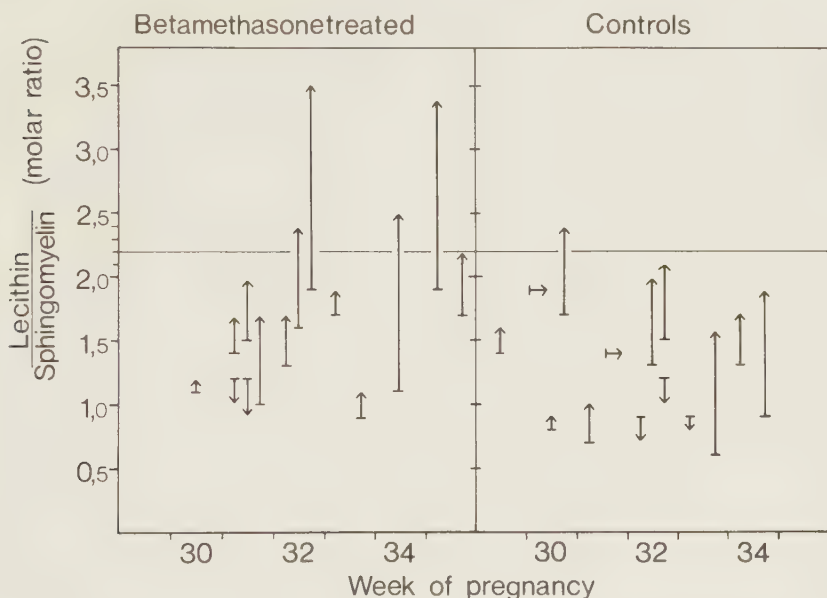


Fig. 7. Individual lecithin/sphingomyelin ratios in amniotic fluid obtained by amniocentesis with four days' interval. The base of the arrow indicates the first value and the head the second.

The patients in the betamethasone-treated group were delivered one to nine weeks after the first amniocentesis, and those in the control group after two to eleven weeks. No infant developed IRDS. In some cases amniotic fluid was obtained at delivery, and the lecithin/sphingomyelin ratio was determined. The lowest value observed at delivery was 2.1.

DISCUSSION

In 1971, Gluck *et al.* reported that respiratory distress syndrome (RDS)* could be "diagnosed" antenatally by determination of the amniotic fluid lecithin/sphingomyelin ratio. They stated that if the ratio was more than 2, the infant would not develop RDS (Borer *et al.*, 1971), but gave no data from cases where the infants had developed this disease. Many investigators (Bryson *et al.*, 1972; Hobbins *et al.*, 1972; Kulkarni *et al.*, 1972; Spellacy and Buhi, 1972; Whitfield *et al.*, 1972; Lemons and Jaffe, 1973; Merola *et al.*, 1974) later stated that a lecithin/sphingomyelin ratio more than 2 indicates satisfactory pulmonary maturity of the fetus.

The present study (I) showed a significantly lower lecithin/sphingomyelin ratio in the nine instances where the infants developed RDS. In seven of them, the ratio was less than 2; in the other two, it was 2.2. The lowest value hitherto observed with our analytical method among newborns not developing RDS is 2.0. Probably, a borderline group exists with a lecithin/sphingomyelin ratio between 2.0 and 2.5.

The validity of values less than 2 in predicting RDS has been questioned, as several investigators have reported absence of the disease despite low lecithin/sphingomyelin ratios (see I, Table IV). This might be due to differences in methodology or delayed delivery

*The term IRDS previously used is replaced by the now more accepted term RDS.

after the samples were obtained. Most investigators include the cold-acetone precipitation step in the extraction procedure, as described by Gluck *et al.* (1971). Significant falls in the lecithin/sphingomyelin ratios after acetone precipitation have been demonstrated (Wagstaff *et al.*, 1974). In the present study (I) all samples were obtained at parturition, and the phospholipid analyses were performed without preceding acetone precipitation. It is also notable that all samples were obtained in a way that avoided contamination with blood or meconium. This is important, as an increase in the lecithin/sphingomyelin ratio has been demonstrated after such contamination (Wagstaff *et al.*, 1974).

The relation between fetal lung maturity and the composition of amniotic fluid phospholipids was further strengthened by the fatty acid analysis of the lecithin fraction. There was a significantly lower proportion of palmitic acid where the infants developed RDS. The results thus indicate that there was a deficiency of dipalmitoyllecithin, the main component of pulmonary surfactant, in those infants that developed RDS. Others have since reported relation between total palmitic acid in the amniotic fluid and fetal pulmonary maturity (Warren *et al.*, 1974).

The present study (I) demonstrates the value of analysing total amniotic fluid phospholipids in order to estimate pulmonary maturity of the fetus, thus predicting the risk of the newborn developing RDS.

The question arises how and to what extent the composition of amniotic fluid phospholipids is related to general fetal maturity.

Arvidson *et al.* (1972) showed that the lecithin/sphingomyelin ratio and the proportion of palmitic acid in the lecithin in amniotic fluid samples obtained during the second trimester were significantly lower than in those obtained at full-term. An abrupt rise in the concentration of amniotic fluid lecithin (Gluck *et al.*, 1971) and of the lecithin/sphingomyelin ratio (Mercus *et al.*, 1973) in the 35th week of pregnancy has been reported. The present study (II) found a progressive increase in the lecithin/sphingomyelin ratio with gestational age, but a considerable range of variation within each week throughout the third trimester. This agrees with results reported by Donald *et al.* (1973). Also the proportion of palmitic acid in the amniotic fluid lecithins increased with advancing gestational age throughout the third trimester and was correlated even more closely to gestational age than the lecithin/sphingomyelin ratio. Others have since found a relation between amniotic lecithin-bound palmitic acid and gestational age (Russel *et al.*, 1974; Das *et al.*, 1975).

According to Gluck *et al.* (1973) the lectihin/sphingomyelin ratio is correlated with gestational age only in normal pregnancies. The present study (II) found no differences between the ratios in complicated and normal pregnancies. This agrees with the findings by Donald *et al.* (1973). Nor did pregnancy complications influence the proportion of palmitic acid in the lecithins.

From the present investigation (II) it can be concluded that particularly the proportion of lecithin-bound palmitic acid and also the lecithin/sphingomyelin ratio of amniotic fluid are related to gestational age by a progressive increase throughout the third trimester. Pregnancy complications did not affect this relation.

Estimate of fetal pulmonary maturity, thus predicting the risk of the infant developing RDS, is of great value in high risk pregnancies where a premature induction of labour may be necessary. However, this is of limited practical value where it is impossible to wait for spontaneous maturation of the fetal lungs. In these cases, it would be valuable to have therapeutic means of accelerating pulmonary maturation.

The observation by Liggins (1969) that lambs born after glucocorticoid-infusion of the ewe were viable at an earlier age than expected and subsequent reports of accelerated lung maturation in lambs and rabbits after administration of glucocorticoids (de Lemos *et al.*, 1970; Kikkawa *et al.*, 1971; Kotas *et al.*, 1971; Motoyama *et al.*, 1971; Wang *et al.*, 1971; Taeush *et al.*, 1972) initiated studies to elucidate the effects of glucocorticoids on human fetal lung tissue (III, IV, V).

The effects *in vitro* were studied in organ culture (III, IV). The lung tissue is thus cultured in its histotypical arrangement (Sorokin, 1961).

Recently, elevated lecithin content and increased lecithin synthesis due to enhanced choline phospho-

transferase activity has been demonstrated in fetal lung homogenates from glucocorticoid-treated rabbits (Farrell and Zachmann, 1973). As the drug was administered *in vivo*, it could not be decided with certainty whether the observed effects were due to other factors, such as an increased level of epinephrin and/or glucose. In the present experiments, direct effects of cortisol on isolated human fetal lung tissue were obtained (III, IV).

Cortisol increased the concentration of phospholipids and the incorporation of radioactively labelled choline into the lecithins (III, IV). This agrees with recent observations by Smith and Torday (1974) and Smith *et al.* (1974), who found enhanced choline incorporation into lecithin of fetal rabbit lung cells cultured under the influence of glucocorticoids. The present study (IV) revealed an increased number of osmiophilic lamellar bodies in the alveolar epithelial cells of human fetal lung tissue incubated with cortisol. This agrees with animal experiments *in vivo* (Kikkawa *et al.*, 1971; Wang *et al.*, 1971). The increased number of osmiophilic lamellar bodies was linked to a concomitant increase in the concentration of lecithins and an increased incorporation of radioactively labelled choline into lecithins.

Osmiophilic lamellar bodies isolated by ultracentrifugation of rat lung homogenate have been shown to contain large amounts of fully saturated lecithins (Gil *et al.*, 1973), the main component of pulmonary surfactant. In the present study (IV) the labelled choline was predominantly associated with the osmiophilic lamellar bodies. This strengthens the hypothesis that these bodies are related to synthesis or storage of pulmonary surfactant.

The effects of cortisol on isolated human fetal lung were already observed at a gestational age of 16 weeks. This is consistent with observations by Ballard and Ballard (1974). They recently reported that, as early as the 14th week of gestation, human fetal lung contained the receptor mechanism necessary for direct responsiveness to glucocorticoids. It has since been shown in cultured fetal rabbit lung cells (Torday *et al.*, 1975) that the binding of glucocorticoids to specific lung cell receptors could really be linked to the action of these hormones on lung maturation.

Although, both morphological and biochemical effects were found at a gestational age of 16 weeks, the effects observed at a gestational age of 20 weeks or more, were more specific. At a gestational age of 16 weeks the relative increments of saturated lecithins, of unsaturated lecithins and of the remaining phospholipids were about the same, but at a gestational age of 20-23 weeks the relative increment of saturated lecithins was greater than that of the other phospholipid fractions (Fig. 3). The incorporation of labelled choline increased relatively more in the saturated lecithins than in the unsaturated lecithins at a gestational age of 20-23 weeks, but not at 16 weeks (Fig. 4).

The results obtained *in vitro* (III, IV) are supported by *in vivo* studies (V) where a significant increase was found in the proportion of palmitic acid in the lecithins of fetal lung tissue obtained during the second trimester after the pregnant woman had received cortisone-acetate for three days.

The close correlation of increased number of osmio-philic lamellar bodies, increased incorporation of radio-actively labelled choline into saturated lecithins, and the accumulation of labelled choline in these bodies in cortisol-treated lung tissue explants strongly indicates that cortisol accelerated the synthesis of pulmonary surfactant in the human fetal lung as early as the second trimester.

This posed the question whether glucocorticoid-induced acceleration of fetal pulmonary maturation is reflected in the amniotic fluid.

Liggins *et al.* (1972) showed a lower incidence of RDS in premature infants delivered after betamethasone treatment of the mother. In a few instances, the lecithin/sphingomyelin ratio of amniotic fluid was determined, but no figures were given. Kling and Kotas (1975) recently reported increased lecithin/sphingomyelin ratios in the baboon after intra-amniotically administered dexamethasone. Spellacy *et al.* (1973) found a higher mean difference between the amniotic lecithin/sphingomyelin ratios before and after dexamethasone-treatment of women in 28th to 32nd week of pregnancy. Caspi *et al.* (1973) reported one instance of premature rupture of the membranes, where the lecithin/sphingomyelin ratio remained unchanged for eight days, but rose sharply after treatment with dexamethasone for three days. Recently Caspi *et al.* (1975) reported a rise in amniotic fluid lecithin/sphingomyelin ratios to values over 2 after dexamethasone administration. Dexamethasone, however, was given in various dosages and administered in different ways; moreover, the interval between pre- and post-treatment

amniotic fluid samples varied from case to case, and they did not report any controls. In the present study (VI), the amniotic fluid samples were obtained identically in both the betamethasone-treated group and in the controls. Betamethasone was administered i.m. in the same dosage to all treated patients, and the second sample of amniotic fluid was obtained after the same interval.

The results show that the amniotic fluid lecithin/sphingomyelin ratio was more than 2.2 in 29 % of the betamethasone-treated patients, and in 7 % of the controls. The level 2.2 was set according to the findings in paper I (see page 17 and 30). The concomitant increase in the percentage of palmitic acid in the lecithin indicates that betamethasone enhanced the synthesis and release of dipalmitoyllecithin. It is notable that betamethasone failed to increase the lecithin/sphingomyelin ratio to more than 2.2 in several cases. The results obtained in III-V, make it plausible that betamethasone stimulated the synthesis of dipalmitoyllecithin in the lung tissue, but that the substance did not reach the amniotic fluid in those cases.

Of the 51 patients, 23 had a lecithin/sphingomyelin ratio more than 2.2 already at the first amniocentesis. In such cases, treatment with glucocorticoids can be avoided, and delivery can take place without any risk of the infant developing RDS.

The present investigation (VI) thus further demonstrates the value of determining the amniotic fluid lecithin/sphingomyelin ratio in high risk pregnancies, where premature delivery is considered or imminent. It also suggests that glucocorticoid-induced acceleration of fetal pulmonary maturation may be reflected in the amniotic fluid as changes in its phospholipid composition.

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